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# Discussion on Validity of *Rana maoershanensis* Based on Partial Sequence of 16S rRNA Gene

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**Abstract** *Rana maoershanensis* found in Mt. Maoershan in Guangxi, China was reported as a new species in 2007, but there was no molecular data for this frog. The partial sequences (543 bp) of 16S rRNA gene from 12 specimens of 3 brown frog species (*Rana hanluica*, *R. maoershanensis* and *R. chensinensis*) were analyzed with 17 specimens of 9 species from GenBank. The nucleotide sequence divergence between *R. maoershanensis* and the other brown frog species were 4.5%–6.5%, with 22–30 nucleotide substitutions at this locus. The phylogenetic relationships based on MP, ML, and Bayesian inference indicate that the brown frogs from Southern China were diverged into three groups (Clades A, B and C). *R. maoershanensis* was clustered together a well-supported subclade (B-1). It is suggested that *R. maoershanensis* is a valid species.

**Keywords** *Rana maoershanensis*, validity, variation, phylogeny, 16S rDNA

## 1. Introduction

Species numbers and taxonomy of brown frogs (*Rana*) from China have changed considerably in the last 20 years. Particularly, two species of *Rana* were reported in Guangxi, China recently, *R. zhenhaiensis* (Zhou *et al.*, 2005) which was known as *R. japonica* (Zhang and Wen, 2000) and *R. maoershanensis* (Lu *et al.*, 2007). The latter was collected from Mt. Maoershan and was first reported as a new distribution record of *R. chaochiaoensis* in Guangxi (Lu *et al.*, 2006), but it was then identified as a new species based on morphological characters (Lu *et al.*, 2007). However, the brown frog from Mt. Maoershan was considered as *R. chaochiaoensis* in Fauna Sinica (Fei *et al.*, 2009). Herein, we report the first molecular data for *R. maoershanensis* based on 16S rRNA and test the validity of recognizing this taxon as a new species.

## 2. Materials and Methods

**2.1 Specimens** The taxonomical scheme proposed by Frost (Frost, 2010) was followed in this study. Twelve specimens of three brown frog species (*R. chensinensis*, *R. hanluica* and *R. maoershanensis*) were analyzed, along with nine species (*R. chaochiaoensis*, *R. longicrus*, *R. omeimontis*, *R. zhenhaiensis*, *R. sauteri*, *R. zhengi*, *R. shuchinae*, *R. Johnsi* and *R. japonica*) from GenBank (Sumida *et al.*, 2003; Roelants *et al.*, 2004; Chen *et al.*, 2005; Frost *et al.*, 2006; Che *et al.*, 2007) (Table 1). These species cover all Chinese brown frogs distributed in Southern China, except for *R. chevronta* Hu and Ye, 1978. In these analyses, we also included the specimens of *R. japonica* based on the previous result that *R. zhenhaiensis* was separated from *R. japonica* and named by Ye *et al.*, (1995). Referring to the results of Jiang *et al.*, (2002) and Che *et al.*, (2007), we chose *Pelophylax nigromaculata* Hallowell, 1861 and *Lithobates catesbeiana* Shaw, 1802 as outgroup taxa.

**2.2 Preparation of DNA, PCR, and DNA sequencing** Genomic DNA was extracted from liver or thigh muscle tissues by using standard phenol-chloroform methods (Sambrook and Russell, 2001). The brown frog samples used were stored in 95% or 100% ethanol at the Liaoning Key Laboratory of Biology Diversity, Shenyang Normal University. Partial sequences of 16S rRNA gene were PCR-amplified and sequenced using the primers F51

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(5′-CCC GCC TGT TTA CCA AAA ACA T-3′) and R51 (5′-GGT CTG AAC TCA GAT CAC GTA-3′) (Sumida *et al*., 2002). The lengths of target sequences were approximately 550 bp segment. PCR amplification was performed in a 25 μL volume reaction with the following conditions: initial denaturation step: 90 s at 94℃, 35 thermo-cycles: denaturation for 45 s at 94℃, primer annealing for 45 s at 55℃, and extension for 90 s at 72℃. Final extension at 72℃ was conducted for 10 min. PCR products were purified and sequenced by Beijing Genomics Institute (BGI).

**2.3 Data analysis** Preliminary multiple alignments were generated by using CLUSTAL W (Thompson *et al.*, 1997) with default parameters, and verified manually. Analysis of genetic variation was performed by MEGA4 (Tamura *et al.*, 2007) and sequence divergences were calculated by Kimura's two-parameter model (Kimura, 1980) with a transition to transversion ratio of 2 : 1. In order to infer the relationships among taxa, the phylogenetic trees

**Table 1** Specimens of brown frogs used in this study and their haplotypes of nucleotide sequences of 16S rRNA gene

| Species and code of specimens | | | Collection locality | Sequence origin/voucher No. | Accession No. |
|---|---|---|---|---|---|
| Ingroup | *R. maoershanensis* | 1 | Mt. Maoershan, Guangxi, China | SYNU08030047 | HQ228161$^{a}$ |
| | | 2 | | SYNU08030061 | HQ228162$^{a}$ |
| | | 3 | | SYNU08030062 | HQ228163$^{a}$ |
| | | 4 | | SYNU08030064 | HQ228164$^{a}$ |
| | | 5 | | SYNU08030068 | HQ228165$^{a}$ |
| | | 6 | | SYNU08030071 | HQ228166$^{a}$ |
| | | 7 | | SYNU08040114 | HQ228167$^{a}$ |
| | *R. hanluica* | 1 | Shuangpai, Hunan, China | SYNU07100490 | HQ228158$^{a}$ |
| | | 2 | | SYNU07100491 | HQ228159$^{a}$ |
| | | 3 | | SYNU07100492 | HQ228160$^{a}$ |
| | *R. chensinensis* | 1 | Shidu, Beijing, China | SYNU07070381 | HQ228156$^{a}$ |
| | | 2 | Qingdao, Shandong, China | SYNU08090671 | HQ228157$^{a}$ |
| | *R. chaochiaoensis* | 1 | Zhaojue, Sichuan, China | GenBank | DQ289107 |
| | | 2 | Zhongdian, Yunnan, China | GenBank | DQ289106 |
| | | 3 | Zhongdian, Yunnan, China | GenBank | DQ289105 |
| | | 4 | Muli, Sichuan, China | GenBank | AF315134 |
| | *R. longicrus* | | Taipei, Taiwan, China | GenBank | AB058881 |
| | *R. omeimontis* | 1 | Hongya, Sichuan, China | GenBank | DQ289108 |
| | | 2 | Mt. Emei, Sichuan, China | GenBank | AF315135 |
| | *R. zhenhaiensis* | 1 | Nanjing, Jiangsu, China | GenBank | AY322279 |
| | | 2 | Nanjing, Jiangsu, China | GenBank | AF315136 |
| | *R. sauteri* | 1 | Kaohsiung, Taiwan, China | GenBank | DQ289109 |
| | | 2 | Taichung, Taiwan, China | GenBank | DQ283124 |
| | *R. zhengi* | 1 | Zhangcun, Sichuan, China | GenBank | DQ289103 |
| | | 2 | Zhangcun, Sichuan, China | GenBank | DQ289104 |
| | *R. johnsi* | | Chi Linh, Vietnam | GenBank | AF206477 |
| | *R. shuchinae* | | Zhaojue, Sichuan, China | GenBank | DQ289126 |
| | *R. japonica* | 1 | Hiroshima, Japan | GenBank | AB058876 |
| | | 2 | Hiroshima, Japan | GenBank | AB058877 |
| Outgroup | *P. nigromaculata* | | Hiroshima, Japan | GenBank | AB043889 |
| | *L. catesbeiana* | | Chengdu, Sichuan, China | GenBank | DQ289127 |

$^{a}$: Sequences new to this study. SYNU = Shenyang Normal University.

were reconstructed using three different methods: maximum parsimony (MP), maximum likelihood (ML) and Bayesian inference (BI). Prior to ML/BI-based phylogenetic analyses, the best-fit substitution model of sequence evolution was chosen on the basis of Hierarchical Likelihood Ratio Tests (hLRT) as implemented in MrModeltest version 2.3 (Nylander, 2004). We used MrBayes version 3.1.2 (Ronquist and Huelsenbeck, 2003) for Bayesian phylogenetic analyses. Four Monte Carlo Markov chains (Yang and Rannala, 1997) were run simultaneously for one million generations. Trees were sampled every 100 generations. The initial 25% trees were excluded as burn-in, leaving 7500 trees for estimating the consensus tree and posterior probabilities distribution. PAUP*4.0b10 (Swofford, 1998) was used to determine the MP and ML trees, the former using a heuristic search with 100 random sequence additions and tree-bisection-reconnection (TBR) branch swapping, and the latter using the substitution model as BI. Bootstrap analyses were performed to examine the confidence of nodes with 1000 replicates for MP and 100 replicates for ML, respectively.

## 3. Results

**3.1 Molecular variation and sequence distances** After alignment, a total of 543 bp were obtained from 16S rRNA gene sequences for further analyses. Among the sites, 88 were polymorphic sites and 58 parsimony informative sites. 20 haplotypes were obtained from 31 specimens of 14 species, including outgroup. There was a haplotype shared among all specimens of *R. maoershanensis*, as well as among the specimens of *R. hanluica*.

Nucleotide sequence divergences based on Kimura's two-parameter model and numbers of nucleotide differences among the 12 brown frog species are shown in Table 2. Both parameters mentioned above were calculated at five levels as follows: (i) between ingroup and the outgroup, sequence divergences ranged between 6.5%–15.6% ($\bar{x}$ = 10.3%), and numbers of nucleotide differences between 30-66; (ii) between ingroup taxa, sequence divergences were 1.2%-6.8% ($\bar{x}$ = 4.6%), with 6–32 nucleotide substitutions; (iii) between *R. maoershanensis* and the other eleven ingroup species, those were 4.5%-6.5% ($\bar{x}$ = 5.6%), with 22-30 nucleotide substitutions; (iv) between *R. maoershanensis* and the five species of *R. longicrus* species group (Fei *et al.*, 2009), sequence divergences were 4.7%–6.2% ($\bar{x}$ = 5.7%), with 22-29 nucleotide substitutions; and (v) between *R. maoershanensis* and *R. chaochiaoensis*, that was 6.2%, with 29 nucleotide substitutions.

**3.2 Phylogenetic analyses** The general time reversible model (GTR) was found by the hLRT to be the best fit model of sequence evolution ($-\ln L$ = 1637.22), with the gamma distribution shape parameter ($\alpha$ = 0.1247) estimated from the data, i.e., the GTR + $\Gamma$-model. We used empirical base frequencies and the substitution model of $\text{rate}_{[A–C]}$ = 2.63, $\text{rate}_{[A–G]}$ = 8.30, $\text{rate}_{[A–T]}$ = 1.78, $\text{rate}_{[C–G]}$ = 0.32, and $\text{rate}_{[C–T]}$ = 19.4, estimated from the data. The Bayesian tree is shown in Figure 1a, and MP strict consensus tree (Length = 220, CI = 0.659, RI = 0.827) is in accordance with ML majority-rule consensus tree (Figure 1b).

Inferred phylogenetic relationships and nodal support

**Table 2** Percent sequence divergences estimated by Kimura's two-parameter model (lower-left diagrams) and numbers of nucleotide differences (upper-right diagrams, transitions / transversions) based on partial sequences of 16S rRNA genes among the brown frog species

| | (1) | (2) | (3) | (4) | (5) | (6) | (7) | (8) | (9) | (10) | (11) | (12) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| (1)*R. chensinensis* | – | 30/8 | 17/5 | 26/10 | 25/6 | 17/9 | 27/7 | 29/8 | 19/8 | 27/9 | 15/10 | 29/10 |
| (2)*R. hanluica* | 8.4 | – | 21/5 | 13/4 | 19/2 | 23/7 | 11/1 | 10/1 | 26/6 | 22/7 | 20/8 | 11/4 |
| (3)*R. maoershanensis* | 4.6 | 5.6 | – | 22/7 | 23/3 | 21/6 | 18/4 | 22/5 | 17/5 | 24/6 | 19/7 | 20/7 |
| (4)*R. chaochiaoensis* | 7.9 | 3.5 | 6.2 | – | 10/4 | 22/9 | 12/3 | 11/4 | 23/6 | 20/9 | 20/10 | 12/5 |
| (5)*R. japonica* | 6.6 | 4.3 | 5.5 | 2.9 | – | 21/5 | 17/1 | 18/1 | 22/4 | 17/5 | 18/6 | 16/4 |
| (6)*R. johnsi* | 5.5 | 6.5 | 5.8 | 6.7 | 5.4 | – | 22/6 | 22/7 | 24/7 | 21/4 | 5/1 | 22/9 |
| (7)*R. longicrus* | 7.4 | 2.5 | 4.7 | 3.2 | 3.7 | 6.0 | – | 6/0 | 22/5 | 21/6 | 19/7 | 4/3 |
| (8)*R. omeimontis* | 8.0 | 2.4 | 5.7 | 3.0 | 4.0 | 6.0 | 1.3 | – | 25/6 | 23/6 | 19/8 | 6/3 |
| (9)*R. sauteri* | 5.6 | 6.8 | 4.5 | 6.3 | 5.5 | 6.6 | 5.7 | 6.5 | – | 23/7 | 21/8 | 24/8 |
| (10)*R. shuchinae* | 7.9 | 6.2 | 6.5 | 6.3 | 4.5 | 5.3 | 5.8 | 6.1 | 6.4 | – | 21/5 | 21/9 |
| (11)*R. zhengi* | 5.3 | 6.0 | 5.5 | 6.4 | 5.0 | 1.2 | 5.5 | 5.6 | 6.1 | 5.6 | – | 19/10 |
| (12)*R. zhenhaiensis* | 8.5 | 3.0 | 5.7 | 3.6 | 4.0 | 6.6 | 1.3 | 1.8 | 6.7 | 6.3 | 6.1 | – |

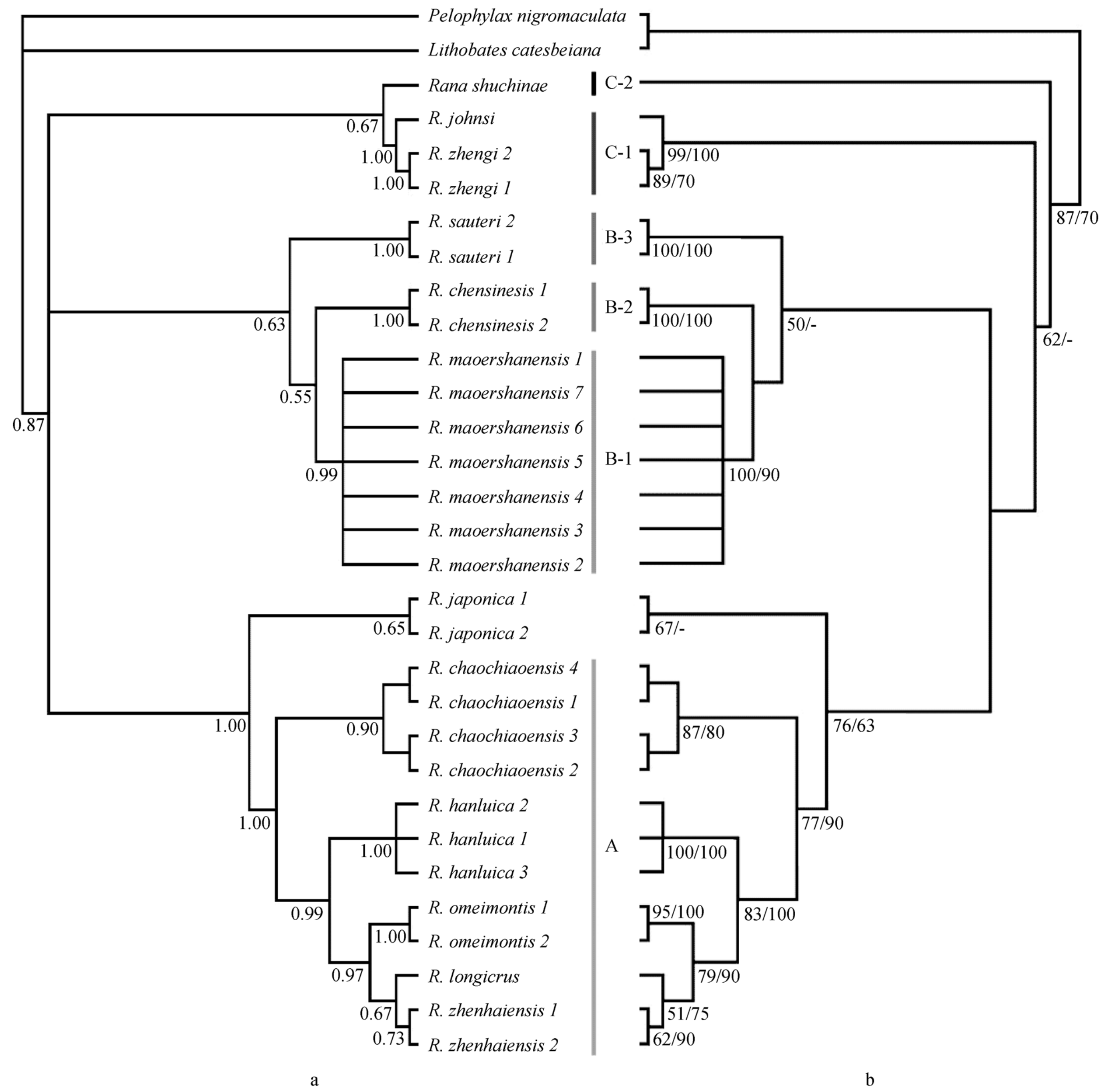

**Figure 1** Gene trees based on 16S rRNA gene fragments resulting from (a) Bayesian 50% majority rule consensus tree and (b) MP strict consensus tree. Support for each node is indicated by single number or trailing paired numbers: (a) Bayesian posterior probabilities; (b) the percent bootstrap support obtained from MP analyses/ML analyses. Maximum likelihood tree topologies were identical to the maximum parsimony tree.

based on MP, ML, and Bayesian analyses of the mitochondrial 16S rRNA gene fragments are almost concordant in their support (Bayesian posterior probabilities, BPP, above 0.90; bootstrap support, BP, above 70%) for the clustering of intra-species, except for *R. japonica*, but show some discrepancies in their estimation of the relationships among species (Figure 1). Mainly, several nodes are unresolved or short with low or intermediate posterior probabilities/bootstrap support values (below 0.90 / 70%).

The three analyses indicated that the ingroup species, excluding *R. japonica,* were divided into three clades (Clades A, B and C), but their relationships remained indistinct. The brown frog species of the *R. longicrus* group (*R. longicrus, R. chaochiaoensis*, *R. omeimontis, R. hanluica* and *R. zhenhaiensis*) were monophyletic (Clade

A), which was strongly supported by posterior probabilities (1.00) and bootstrap (77% and 90% support for MP and ML bootstrap values, respectively). *R. japonica* from Japan was clustered with *R. longicrus* group (1.00, 76% and 63% support). Clade C consisted of *R. zhengi*, *R. johnsi* and *R. shuchinae*, which were divided into two subclades (C-1 and C-2). Subclade C-1 including *R. zhengi* and *R. johnsi* was strongly supported by posterior probabilities (1.00) and bootstrap (99% and 100%). In Clade B, *R. maoershanensis* (Clade B1) was clustered with *R. chensinensis* (Clade B2) and *R. sauteri* (Clade B3), but the relationships with low support (0.63 and 50% support for posterior probabilities and MP bootstrap, respectively). The Bayesian tree that was multifurcating differed in topology from MP and ML trees. The seven specimens of *R. maoershanensis* were clustered together (Clade B1, 0.99, 100%, 90% support), and were positioned inside Clade B.

## 4. Discussion

The brown frog species clustered into three species groups (Fei *et al.*, 2009), the *Rana longicrus* species group, *R. chensinesis* species group and *R. amurensis* species group, respectively. *R. longicrus* species group (*R. longicrus, R. chaochiaoensis*, *R. omeimontis, R. hanluica*, *R. zhenhaiensis* and *R. chevronta*) known as Southern species, are distributed over the Oriental realm. *R. maoershanensis* is found in Mt. Maoershan, Guangxi, which is part of the Oriental realm, and we selected *R. longicrus* species group for comparative studies with *R. maoershanensis*. Both *R. chensinesis* and the species of *R. longicrus* species group overlap in their geographic ranges, thus two specimens of *R. chensinesis* from Shidu, Beijing and Qingdao, Shandong were analyzed.

Mitochondrial genes have been used successfully for amphibian phylogenetic studies (Tanaka-Ueno *et al.*, 1998; Sumida *et al.*, 2002; Darst and Cannatella, 2004; Li *et al.*, 2004; Kurabayashi *et al.*, 2005; Che *et al.*, 2007; Jang-Liaw and Lee, 2009). The magnitude of sequence divergences of mitochondrial DNA are correlated to taxon divergence. Kurabayashi *et al.* (2005) concluded that the sequence divergence of 16S rDNA from ranid frogs was below 2.21% between the specimens of intra-species and the sequences divergences ranged from 2.21% to 10% between inter-species. The results from Jiang and Zhou (2001 a, b) indicates that the sequence divergences based on mitochondrial 12S rDNA from *Rana* frogs ranged from 0 to 9.20% between inter-species. In our study, the sequences divergences based on 16S rRNA gene fragments between *R. maoershanensis* and the remaining eleven ingroup species ranged between 4.5%–6.5% ($\overline{x}$ = 5.6%), and those between *R. maoershanensis* and *R. chaochiaoensis* being 6.20% (Table 2). The results were found in accordance with those of the study by Kurabayashi *et al.* (2005) and Jiang and Zhou (2001).

The phylogenetic tree (Figure 1) shows that the members of the brown frog species from Southern China diverged into three groups (Clades A, B and C). Our molecular data clearly indicated that the monophyly (Clade A) of the *R. longicrus* species group had significant statistical support (BPP = 1.00), and that *R. maoershanensis* differed greatly from the species complex of the *R. longicrus* group. *R. maoershanensis*, *R. chensinesis* and *R. sauteri* formed Clade B. In external and anatomical characters, such as dorsalateral folds, head shape and labial tooth row formula (LTRF), *R. maoershanensis* differed from other brown frog species (Lu *et al.*, 2007). Our present results using 16S rRNA gene sequences support the views of morphological classification, though the relationship between *R. maoershanensis* (Clade B-1) and other brown frogs remained unresolved.

On the basis of molecular data and morphological characters (Lu *et al.*, 2007), we therefore confirm that *R. maoershanensis* is a distant relative of *R. longicrus* species group, as well as *R. chaochiaoensis* specially. *R. maoershanensis* merit specific taxonomic position, i.e., *R. maoershanensis* is valid species. However, more thorough and multilocus study may be required to strengthen the preliminary results presented in this paper.

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